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Retrospective View of the Value of Short-Term Genetic Bioassays in Predicting the Chronic Effects of Diesel Soot

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16. ABSTRACT

In retrospect, it is now safe to conclude that short-term mutagenicity assays were not only useful but instrumental in: (1) indicating that diesel soot was potentially carcinogenic and should be evaluated in chronic animal cancer bioassays, (2) identifying NO_2-PAHs as potential carcinogens in this very complex mixture, (3) providing initial evidence that the mutagens were bioavailable, and (4) estimating the relative importance of various sources and fuels and other factors which can influence human exposure to carcinogens. This is not to say that short-term bioassays used alone can accomplish all of this. However, used in combination with chemical/analytical methods and toxicological tools, short-term genetic bioassays have become a critical component of many environmental health studies. Although substantial advances in our knowledge of the toxicology of diesel emissions have been made since 1978 when the initial observation that the organics extracted from diesel soot were mutagenic, a number of important questions remain not only for diesel emissions but for other combustion sources as well. Are the chemicals which induce positive results in the short-term bioassays the same agents which cause tumors in chronic animal bioassays? Which phase of the diesel emissions (gaseous or particulate) is carcinogenic in the animal inhalation studies? With advances in our understanding of the molecular mechanisms involved in producing chronic effects such as cancer, it is possible that new genetic tools and short-term bioassays will continue to contribute to our ability to answer these and other questions as they arise.

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A RETROSPECTIVE VIEW OF THE VALUE OF SHORT-TERM GENETIC BIOASSAYS IN PREDICTING THE CHRONIC EFFECTS OF DIESEL SOOT

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The organic matter extractable from diesel soot particles was first reported to be mutagenic in bacteria in 1978 (1-3). This finding was rapidly confirmed by many groups (4-6). These organics were also found to cause gene mutations, DNA damage, and chromosomal effects in several mammalian cell systems (7-11). Bioassay directed fractionation and characterization studies using bacterial mutagenicity assays indicated that compounds more polar than polynuclear aromatic hydrocarbons (PAHs) were responsible for most of the mutagenicity in these organics (1, 4-6, 12). By 1982 a number of potent nitrated PAHs (NO_2-PAHs) had been identified in diesel soot (13-16).

Short-term genetic bioassays were used in studies designed to determine the bioavailability and metabolism of diesel soot mutagens (4, 17, 18). Concern that bacterial mutagenesis assays may "overestimate" the mutagenic activity of diesel soot due to the presence of NO_2-PAHs, led to studies on the mammalian metabolism and DNA-binding of NO_2-PAHs alone and associated with diesel soot. These studies showed that the bacterial mutagens and NO_2-PAHs were rapidly released from diesel particles. Metabolism studies in both whole animals and mammalian target cells (e.g., tracheal cells) have demonstrated that NO_2-PAHs are metabolized by both oxidative and reductive pathways to produce metabolites that bind covalently to DNA (19-21). In diesel soot extracts, the concentration of certain NO_2-PAHs is highly correlated with the mutagenicity and tumor-initiating activity of the extracts (22). Soots from many other combustion sources (e.g., wood stoves and gasoline automobiles) contain substantially less NO_2-PAHs than diesel emissions (15).

Short-term genetic bioassays have been used in a series of studies designed to determine the comparative potency and characteristics of various diesel soot extracts compared to other combustion sources (7, 23, 24). Bacterial and mammalian cell assays have been compared to mouse skin tumor initiation assays. These studies suggest that the relative potency of diesel soot extracts in bacterial or mammalian cell assays can be used to predict the relative carcinogenic potency in rodent assays and in humans under certain conditions and assumptions. Comparative studies of the mutagenic emission rates of various automobiles and fuels provide a simplified method for directly comparing alternative sources and technologies.

Studies to evaluate the mutagenicity of whole diesel emissions containing both the soot particles and gases have been conducted in plants, insects, and mammals (8, 25). Bacterial assays were used to evaluate the gaseous organics that could be collected by adsorption or condensation. These studies demonstrate that the gaseous components also contain mutagens. In vivo mutagenesis studies of rodents after relatively short inhalation exposures, however, show much less mutagenicity and demonstrated no heritable effects (25). This suggests that either the mutagenic components of diesel emissions did not reach the gonads under these exposure conditions, that these assays are insensitive to the mutagens present in diesel emissions, or the effects were below the level of detection.

Incomplete combustion of many types of fuels result in the production of soot. The International Agency for Research on Cancer's (IARC) Monograph on Soot (26) concludes that there is sufficient evidence that soot is carcinogenic to humans. Very few studies of humans exposed to diesel soot, however, have clearly shown evidence of increased cancer risk. Many soot extracts have also been shown to be carcinogenic in experimental animals; however, few rodent inhalation studies of combustion emissions have demonstrated carcinogenicity. New studies, reported in this volume, showing that diesel emissions are carcinogenic to rodents after chronic inhalation are consistent with the IARC conclusion that soot is a human and animal carcinogen. These new results are also consistent with the positive short-term genetic bioassay results reported over five years earlier. In this paper we take a retrospective view of the short-term genetic bioassay data developed over the past eight years and their value in predicting chronic carcinogenic effects.

WHAT WERE THE EARLY BIOASSAY CLUES REGARDING THE RISK OF DIESEL SOOT?

We found the extractable organics (tar) from diesel particle emissions to consistently cause positive responses in short-term mutagenesis and carcinogenesis bioassays (8, 11) as summarized in Table I. Other investigators have reported similar samples to be mutagenic in human cells (9, 27). We have recently evaluated the genetic activity (potency) data from diesel and gasoline automotive emissions in a number of bioassays using the genetic activity profile method of Waters (28, 29). These profiles provide a graphic representation of bioassay data that facilitates both visual and computer-assisted comparative assessment. The concept of a genetic activity profile for a chemical originated from the need to represent in a single two-dimensional configuration the qualitative and quantitative data from a large number of genetic bioassay systems (currently more than 200). The x-axis values of the profiles shown here (Fig. 1) represent the bioassays in an endpoint/phylogenetic sequence, and the y-axis values represent

TABLE I

SUMMARY OF THE RESULTS OF SHORT-TERM BIOASSAYS USED IN THE EVALUATION OF DIESEL PARTICLE EXTRACTS

Assays	Genetox Code	Results for Diesel Particle Organics
Mutagenesis Bioassays		
Gene Mutation Assays		
Bacterial		
Salmonella typhimurium	SA9	+
Escherichia coli WP2	EC2	+
Escherichia coli K12	ECK	+
Mammalian cell		
Mouse lymphoma, L5178Y/TK$^{+/-}$ locus	G5T	+
Chinese hamster ovary, CHO, HGPRT locus	GCO	+
Chinese hamster lung, V79, HGPRT locus	G9H	(-)
Mouse embryo fibroblasts, Balb/c 3T3, Oua^{r}	GIA	(+)
DNA Damage Assays		
Yeast		
Saccharomyces cerevisiae		
D3 mitotic recombination assay	SC3	(+)
D3 preincubation mitotic recombination assay	SCP	-
D4 mitotic gene conversion assay	SCG	-
D7 induced mitotic crossing over	SCH	(+)
D7 reverse mutation	SCR	-
D7 gene conversion assay	SCG	-
Mammalian Cell		
Unscheduled DNA repair in liver cells	UPR	+
Sister chromatid exchanges in CHO cells	SIC	+
DNA strand breaks in SHE cells	DIA	-
Chromosomal Aberrations		
Mammalian Cells		
CHO cells	CIC	+
Human lymphocytes	CHL	(+)
Carcinogenesis Bioassays		
Oncogenic Transformation Assays		
Chemical Transformation		
Mouse embryo fibroblasts, Balb/c 3T3	TBM	(+)
Mouse embryo fibroblasts, C3H10T1/2	TCM	-
Syrian hamster embryo, SHE, focus assay	TFS	-
Viral Enhancement of Transformation		
SA7 virus enhancement in SHE cells	TEV	+
Skin Tumor Initiation		
SENCAR mice	SKT	+
C57 Black mice		(-)
C3H/HeJ mice		(-)

() indicates the response was either: weak, not reproducible, or was observed in only one sample

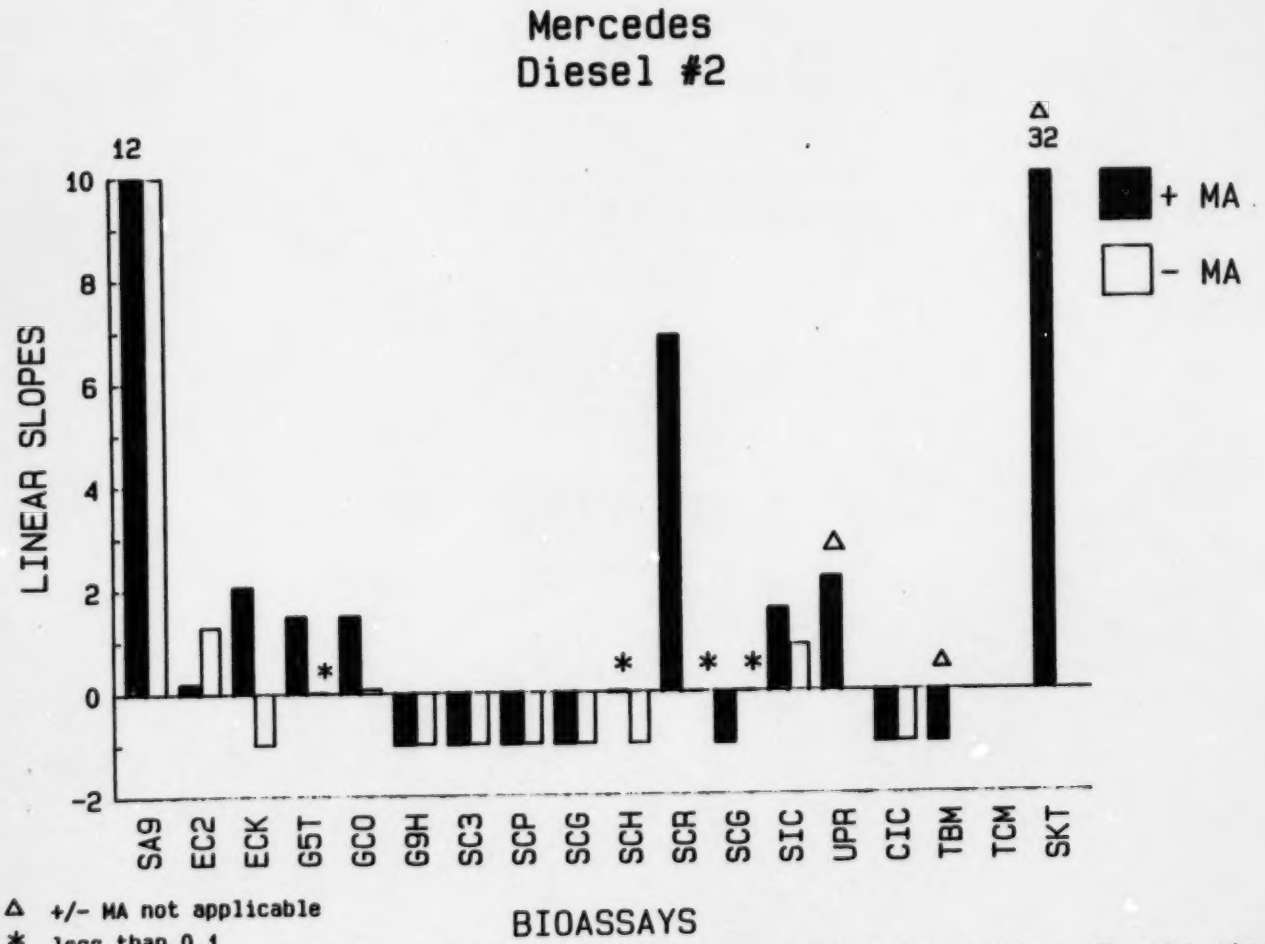

Fig. 1. Genetic activity profile of the extractable organics from diesel particles emitted from a Mercedes automobile.

the potency or linear slope of the dose response.

These results all suggest that diesel soot contains mutagens and carcinogens and that inhalation of diesel soot is very likely to pose an excess cancer risk to whole animals and humans. The negative results reported in the first several inhalation carcinogenesis studies (30-33) led to questions about the use of short-term bioassays and inhalation studies such as: 1) Were the carcinogens associated with the particles not bioavailable? 2) Did the short-term mutagenesis bioassays "overestimate" the potential carcinogenicity of the diesel emissions? 3) Were there mitigating factors present in the whole emissions that had anti-carcinogenic properties? or 4) Were the animal inhalation models or study designs employed simply inappropriate?

Several of these questions were addressed by using genetic bioassay methods as described below.

ARE THE MUTAGENS BIOAVAILABLE?

Initial studies reported that the mutagens associated with diesel particles were not "bioavailable" when incubated with lung fluids or other physiological fluids (34, 35). Subsequent studies demonstrated that the mutagens and specific components (e.g. 1-nitropyrene) were removed from particles after incubation with lung fluids and lung cells (36, 17); however, the proteinacious fluids were directly antimutagenic by a protein binding mechanism in the Ames assay (36). Other investigators have demonstrated that the antimutagenic activity of S9 with diesel extracts was non-enzymatic (37).

A number of studies have been reported that directly assess the mutagenicity and bioavailability of mutagens from whole particles. Cultured cells attached to a surface will readily engulf small particles; and in two independent studies, whole diesel particles induced mutations in CHO cells (38) and human fibroblasts (27). The most convincing studies on the bioavailability of mutagens from diesel particles have been conducted in vivo either by inhalation or intratracheal instillation. The inhalation studies (39-41) are described in more detail in this volume; however, they generally support the bioavailability of mutagens observed in the short-term bioassay studies. We have found that ^{14}C-nitropyrene vapor coated onto diesel particles and intratracheally instilled into rats was readily released from the particles both in the lung and GI tract (18).

WHAT ARE THE MUTAGENS IN DIESEL SOOT?

The discovery that organics from diesel soot were mutagenic (1) resulted from studies to fractionate and chemically characterize the mutagenic constituents. We initially used a fractionation scheme developed by Swain et al (42) for cigarette smoke condensate. The moderately polar and highly polar fractions contained

most of the bacterial mutagenic activity as shown in Table II. The moderately polar fraction, which was the most mutagenic in Salmonella, induced mutation and oncogenic transformation in Balb/c-3T3 cells but did not induce mitotic recombination in *Saccharomyces cervevisiae* D3 (Table III). The fraction which contains the most polar and highly oxygenated species, was also mutagenic in mammalian cells in the absence of S9 and induced mitotic recombination and oncogenic transformation. Conventional gas chromatography/mass spectroscopy identified many fluorenones and methylated fluorenones as major constituents of these mutagenic fractions. None of these or other identified constituents accounted for the direct-acting frameshift mutagenic activity observed. Studies with nitroreductase-deficient strains of *Salmonella typhimurium* showed a substantial reduction in the mutagenicity suggesting that nitrated com-pounds contributed to this direct-acting mutagenicity (43). Nitrated polycyclic aromatic hydrocarbons (NO_2-PAHs) are potent direct-acting frameshift mutagens initially detected in xerographic toners (44). A series of NO_2-PAHs were later identified and quantitated in diesel extracts in order to estimate their contribution to the mutagenic activity of diesel particulate emissions (15, 45). These studies showed that NO_2-PAHs, di-NO_2-PAHs, and hydroxy-NO_2-PAHs together account for much of the mutagenicity observed in *Salmonella typhimurium*. Particulate emissions from catalyst-equipped gasoline-engine vehicles using unleaded fuel contain significantly less of these NO_2-PAHs (15). The mutagenic activity of both leaded- and unleaded-gasoline emissions is substantially increased with the addition of an exogenous metabolic activation (MA) system, suggesting that the unsubstituted PAHs may play a more important role than do NO_2-PAHs in the mutagenicity and carcinogenicity of gasoline emissions (8, 11).

Although NO_2-PAHs were identified in extracts of diesel particles and urban air particulate matter, the quantification of these compounds at low levels has posed problems for analytical chemists because the conventional analytical techniques for quantifying PAHs are relatively insensitive to nitro-substituted PAHs. The dinitropyrene isomers are so highly mutagenic in the Ames (TA98 -S9) assay that trace concentrations of these compounds, if present, could account for a major proportion of the observed mutagenic activity. Therefore, a capillary column GC/MS analytical technique using on column injection and negative chemical ionization (NCI) detection was developed to detect these and other nitro-PAHs at very low concentrations (15). This technique was applied to extracts of soot particles from diesel and gasoline vehicles and urban air particles. Over twenty different NO_2-PAHs were identified in the diesel engine extracts. 1-Nitropyrene was the NO_2-PAH detected in greatest quantity in the diesel extracts (107-1590 ppm relative to the weight of the extract), followed by the nitrophenanthrene/anthracene isomers. The only

TABLE II

DISTRIBUTION OF THE MASS AND BACTERIAL/MUTAGENIC ACTIVITY OF FRACTIONATED DIESEL PARTICLE ORGANICS

Fraction	Mass (%)	Specific Mutagenic Activity (rev/mg)		Distribution of Mutagenic Activity (%)	
		-S9	+S9	-S9	+S9
Organic acids	14.9	193	248	4.9	9.5
Organic bases	0.3	43.8	132	0.02	0.10
Ether insolubles	3.9	53.9	80.9	0.36	0.80
Paraffins	36.7	Neg.	Neg.	0.0	0.0
Aromatics	6.9	49.5	30.1	0.60	0.54
Moderately Polar	5.0	7520	2620	64.9	33.5
Highly Polar	26.9	629	798	29.2	55.4
Unfractionated (DCM)	-	2557	1625		

TABLE III

COMPARISON OF THE BIOASSAY ACTIVITY OF THE TOTAL ORGANICS AND TWO FRACTIONS IN FIVE SHORT-TERM BIOASSAYS

Bioassay	Total Extract[a]		Moderately Polar Fraction		Highly Polar Fraction	
	-S9	+S9	-S9	+S9	-S9	+S9
Salmonella typhimurium (revertants/µg)	2.6	1.6	7.5	2.6	0.6	0.8
Mitotic Recombination in Saccharomyces cerevisiae D3 (mitotic recombination/ µg/ml x10^{-6})	0.3	0.1	Neg.	Neg.	0.2	0.5
Gene Mutation in L5178Y Mouse Lymphoma Cells (mutation frequency/ µg/ml x 10^{-6})	NT		NT		5.7	0.7
Gene Mutation in Balb/c 3T3 Cells (mutation frequency/ µg/ml x 10^{-6})	0.6	0.05	1.2	1.6	1.6	1.4
Oncogenic Transformation in Balb/c 3T3 Cells (transformation frequency/ µg/ml x10^{-5})	0.3	0.06	1.4	0.6	0.8	1.0

aTotal extract and fractions are those shown in Table II.

dinitro-PAHs for which analytical standards were available, the dinitropyrene isomers, were detected in one diesel extract sample at sub-ppm concentrations (0.4-0.6 ppm).

NO_2-PAHs, ARE THEY THE CULPRITS?

Quantification of the concentration of the NO_2-PAHs in diesel soot and determination of their contribution to the direct-acting mutagenicity in *Salmonella typhimurium* TA 98 (Table IV) shows that although 1-nitropyrene was present at the highest concentration (107-1590 ppm) in the diesel particle extracts, it accounted for only 3-13% of the mutagenicity. 3-Nitrofluoranthene present at 1 ppm to 7 ppm in the diesel samples accounted for 0.8% to 1.4% of the mutagenicity. By using the mutagenicity values determined in separate experiments for 2-nitrofluorene and those reported in the literature for 1-nitronaphthalene, these compounds were estimated to account for less than 0.01% of the mutagenic activity. Although the dinitropyrene isomers (1,3; 1,6; and 1,8) were detected in only one diesel sample (Auto 2) at 0.4-0.6 ppm (sum of 1.6 ppm), their mutagenic activity (496,000; 629,000; and 870,000 rev/mg, respectively) was high enough to account for 26% of the mutagenicity of this sample. The total "direct-acting" mutagenic activity in *Salmonella typhimurium* TA98 that can be accounted for by the 23 nitro-PAHs quantified in Diesel 2 is 40%. This estimation is supported by the loss of 50% of the mutagenic activity of this extract when it was assayed in TA98NRD a classical nitroreductase-deficient *Salmonella typhimurium* tester strain obtained from H. Rosenkranz (49).

The fact that dinitropyrenes at concentrations below the ppm level can account for nearly one-third of the mutagenic activity (50) suggests that the presence of other highly potent dinitro-PAHs may account for even more of the mutagenic activity in the moderately polar neutral fraction. A recent application of S. typhimurium tester strains developed to exhibit resistance to dinitropyrenes (e.g., TA98/1,8DNP$_6$) has led to even larger estimations of the contributions of dinitropyrenes to the mutagenicity of diesel particle extracts. The lack of quantitative data on the concentrations of the dinitropyrene isomers in such samples has previously made it impossible to confirm whether the concentrations of the dinitropyrenes are indeed sufficient to account for the contribution predicted by the resistant tester strains. Pederson (51) has reported that in several light-duty diesel particle extracts, 50% to 90% of the TA98 (-S9) mutagenicity is lost when the extracts are tested in TA98/1,8DNP$_6$. Because these strains may show a resistance to other dinitro-PAHs, it is possible that highly potent dinitro-substituted isomers of other parent PAHs present in the particle extracts may also contribute to the mutagenicity of the moderately polar neutral

TABLE IV

CONTRIBUTION OF NO_2-PAHS TO THE MUTAGENIC ACTIVITY OF PARTICLE EXTRACTS IN SALMONELLA TYPHIMURIUM TA98 (-S9)

Extract Sample	Mutagenic Activity (rev/µg) TA98 -S9	1-nitropyrene (ppm)	(%)[a]	nitrofluoranthene (ppm)	(%)	dinitropyrene isomers (ppm)	(%)
Diesel							
Auto 1	13.	1590	11.	7.0	1.4	--	--
Auto 2	3.9	589	13.	1.2	0.8	1.6	26.
Auto 3	3.5	107	2.7	0.9	0.8	--	--
Gasoline							
Auto 4	1.6	2.5	0.1	--	--	--	--

[a]Percent contribution to the total mutagenicity in Salmonella based on the concentration and mutagenicity of the individual NO_2-PAH.

TABLE V

NITROPYRENE AND NITROFLUORANTHENE CONCENTRATIONS IN DIESEL PARTICLE EXTRACTS AND CORRELATION ANALYSIS WITH MUTAGENIC AND TUMORIGENIC ACTIVITY

Particle Extract	1-NP[a] ppm	3-NF[b] ppm	Ames TA98 (-S9) rev/µg[c]	Mouse Lymphoma (-S9) MF/µg/ml[d]	Tumor Initiation pap/mouse/µg[e]
Diesel Auto 1	1590	7.0	13.0	4.2	590
Diesel Auto 2	589	2.9	3.9	0.98	240
Diesel Auto 3	107	1.2	3.5	1.2	310
Gasoline Auto 4	2.5	0.9	1.6	0.38	170
Correlation Coef.					
r^2 with 1-NP			0.91	0.98	0.82
r^2 with 3-NF			>0.99	0.99	0.95

[a]1-Nitropyrene
[b]3-Nitrofluoranthene
[c]Revertants per µg
[d]Mutation frequency (mutants per 10^6 survivors) per µg per ml
[e]Papillomas per mouse per µg in SENCAR mice

fraction.

Good correlations (r^2>0.90) were observed when the slope of the dose-response for the mutagenic activity of this same seies of automotive particle extracts in S. typhimurium strain TA98(-S9) was plotted versus the mutagenic activity in the mammalian cell assays and the skin tumor initiating activity. The correlation of mutagenic and skin tumor initiating activity with the concentration of selected nitro-PAHs was examined for these diesel and gasoline samples. Table V shows the high correlations observed between the concentrations of 1-nitropyrene and 3-nitrofluoranthene and the mutagenic activity in *Salmonella typhimurium* (-S9), L5178Y mouse lymphoma cells (-S9), and skin tumor initiating activity in SENCAR mice. The r^2 correlation coefficient in the presence of S9 (not shown) was somewhat lower. The mutagenicity and tumor initiation activity (-S9) also correlated well (r^2>0.9) with the concentrations of the nitro-252 isomer, the nitro-228 isomers, nitromethylpyrenes, and nitrofluorenes; while no correlation was observed with several of the other less mutagenic, lower molecular weight nitro-PAHs (nitro-phenanthrenes and nitronaphthalenes). The quantified mono-NO_2-PAHs account for less than 20% of the direct-acting bacterial mutagenicity of these samples. The high correlations observed between the concentration of these compounds and the mutagenic activity of the total extract, therefore, suggest that the unidentified mutagens responsible for the remainder of the mutagenic activity and possibly the mutagenic and carcinogenic activity in other bioassays is directly related to the relative concentrations of these mono-NO_2-PAHs. Because the remaining unidentified mutagens appear to be located primarily in the chemical fractions that are more polar than the fraction that contains the unsubstituted and mono-NO_2-PAHs, it is possible that other di-NO_2-PAHs (e.g., dinitrofluoranthenes) or other oxygenated NO_2-PAH's (e.g., hydroxy-nitro-PAHs) species are responsible for the unidentified mutagenic activity.

ARE THE ORGANICS FROM DIESEL SOOT SIMILAR TO OTHER COMBUSTION ORGANICS?

Characterization of the mutagenicity of emissions from other combustion sources shows some general similarities (46). In both wood and diesel combustion, 82-99% of the mutagenicity was in the neutral fraction. Very little mass or mutagenic activity was observed in the organic bases. Differences between these sources were observed in the distribution of mass and mutagenicity in the neutral subfractions.

Bioassay-directed fractionation and chemical characterization studies also have been used to characterize and compare the complex organic emissions from roofing tar pots, coke ovens, and cigarette smoke. To obtain a gross characterization of the chemical classes present in the samples, the chemical class distribution was determined by solvent partitioning the organics into acidic, basic, neutral, and

cyclohexane insoluble fractions. The neutral fractions were further separated into the nonpolar neutrals, aromatics (nitro-methane soluble) and polar neutrals. Characterization of the distribution of bacterial mutagenic activity (Ames *Salmonella typhimurium* bioassay) in each of these fractions (47, 48) showed significant differences between the diesel, coke oven main, roofing tar, and cigarette smoke condensate samples as shown in Fig. 2. In the diesel samples, over 90% of the mutagenic activity was located in the aromatic and polar-neutral fractions, and a significant portion of this activity can be accounted for by NO_2-PAHs. The cigarette smoke condensate, coke oven main, and roofing tar samples did not contain detectable amounts of NO_2-PAHs (48). Most of the mutagenicity of coke oven main sample was found in the basic fraction (37%) and polar neutral fraction (39%). The cigarette smoke condensate sample also had significant activity in the basic fraction (66%), but chemical analysis indicated that the components differed significantly from those of the coke oven main sample. The roofing tar sample contained aromatic (14%) and polar (75%) mutagenic con-stituents that were not NO_2-PAH's. The PAH subfraction of each of these samples accounted for only a small portion of the mutagenicity [e.g. diesel (0.2%), cigarette smoke condensate (0.1%), roofing tar (5%), and coke oven main (8%)].

Although the specific mutagens in these different sources are not identical, they all cause frameshift mutations and appear to be compounds that could be classified as polycyclic organic matter. Chemical characterization suggests that in addition to nitrated NO_2-PAHs found in the slightly and moderately polar neutrals, hydroxylated and carboxylated polycyclic organics are found in the organic acid fraction, aromatic amines and nitrogen heterocycles are found in the organic bases, and highly oxygenated quinones, diones, and nitro-oxygenated compounds are found in the polar neutral fractions.

CAN GENETIC BIOASSAYS BE USED TO ESTIMATE RISK FROM NEW ENGINES, FUELS OR CONTROL TECHNOLOGIES?

A comparative potency method has been developed for cancer risk assessment of diesel particle emissions based on a constant relative potency hypothesis and using data from a battery of short-term mutagenesis bioassays and animal tumorigenicity studies on a series of diesel vehicles emissions (23,24). These same bioassays were used to evaluate three complex emissions for which human lung cancer risk estimations were available (emissions from coke ovens, roofing tar pots, and cigarette smoke).

The comparative potency method for cancer risk assessment is based on the hypothesis that there is a constant relative potency between two different carcinogens (C1 and C2) across different bioassay systems (B1 and B2). This

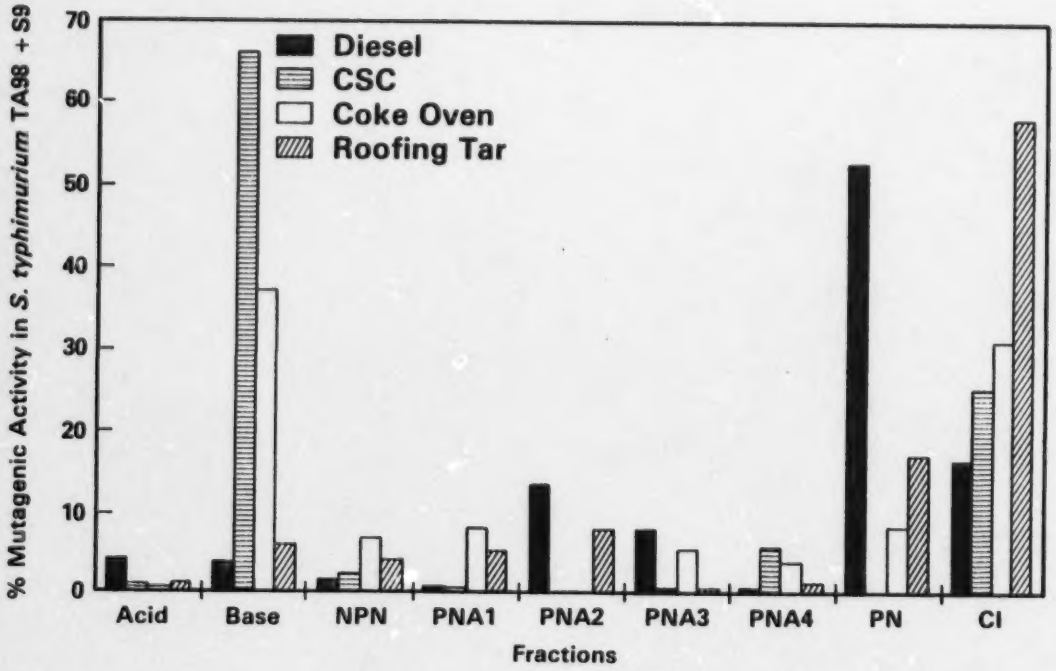

Fig. 2. Distribution of mutagenic activity across fractions separated by liquid partitioning (48). Fractions are as follows: organic acids (Acid), organic bases (Base), nonpolar neutral-alphatics (NPN), polynuclear aromatic hydrocarbons (PNA1), moderately polar fractions separated from the PNA fraction (PNA2, PNA3, PNA4), polar neutrals (PN) and highly polar cyclohexane insoluables (CI).

constant relative potency assumption has been tested most extensively for diesel emissions where the largest series of emissions has been tested in several mutagenesis and carcinogenesis bioassays. The test of this model is whether there is a constant relationship (k) between the relative potencies in the two bioassays being compared such that:

$$\frac{\text{Relative Potency (C1/C2) in Bioassay(1)}}{\text{Relative Potency (C1/C2) in Bioassay(2)}} = \text{constant (k)}$$

Based upon the data avialable on diesel emissions, it appears that this assumption holds when comparing the relative potency in the Ames *Salmonella typhimurium* (TA 98) assay, the mouse lymphoma assay,the sister chromatid exchange assay in CHO cells, and mouse skin tumor initiation in SENCAR mice. A number of short-term bioassays that did not result in quantitative dose-response data or in which the responses were very weak could not be evaluated.

In order to adequately test this hypothesis for prediction of human cancer risk, a more extensive comparative data base is needed for human lung cancer. This hypothesis was tested for only the three complex organic emissions from a coke oven, roofing tar pot, and cigarettes by using human lung cancer data from epidemiological studies of humans exposed to these emissions and testing these emissions in a series of short-term mutagenesis bioassays and animal tumorigenesis assays (23,24). We are currently in the process of expanding this data base in a cancer epidemiology and comparative bioassay study in Xuan Wei province in China (52). Although pure compounds have been reported to range in carcinogenic and mutagenic potency over six orders of magnitude (53), the relative potency of combustion emissions ranged from only a five-fold difference in the mouse skin tumorigenesis assay to a 150-fold difference in the mouse lymphoma gene mutation assay. The Ames *Salmonella typhimurium* mutagenesis assay showed approximately two orders of magnitude difference (100-110 fold) in mutagenic activity from the weakest to the most potent emission sample when measured per mass of extractable organics (e.g., rev/ug). The emission rates (rev/km or rev/J) for the particle-bound organics, however, differed on some cases by as much as five orders of magnitude (10^5). When attempting to directly compare the mutagenicity of emissions from various sources, it is critical to compare the rates of mutagenic emissions on the same basis (e.g., fuel consumption, distance driven, energy consumption, or yield). To compare rates of mutagenic emissions of mobile sources, we have compared the mutagenic activity per kilometer, as shown in Table VI.

This comparative potency method provides a framework for evaluating alternative energy sources, fuels, or control technologies by comparing them to the conventional technologies in short-term genetic bioassays. It is possible to extend the method described here for estimating lung cancer unit risks for diesel and

TABLE VI

COMPARATIVE MUTAGENIC AND TUMORIGENIC EMISSION RATES

Vehicles[a]	Organic Emission Rate	Potency of Organics[b]				Activity Emission Rates[c]			
		Ames TA98 (+S9)	Mouse Lymph. (+S9)	SCE CHO (+S9)	Skin Tumor Init.	Ames TA98 (+S9)	Mouse Lymph. (+S9)	SCE CHO (+S9)	Skin Tumor Init.
	(mg/km)					($\times 10^5$)	($\times 10^4$)	($\times 10^3$)	
Diesel									
Car (Mercedes)	20.2	12.0	1.5	0.16	0.37	2.4	3.0	3.2	7.5
Car (VW Rabbit)	52.2	6.1	0.72	0.03	0.24	3.2	3.8	1.6	12.5
Truck (Ford/Cat)	312.0	1.7	0.29	----		5.3	8.5	---	
Bus (GM)	362.0	0.1	0.35	----		.4	13.0	---	
Gasoline									
Non-catalyst (Ford Van)	5.61	32.0	5.7	0.47	0.20	1.8	3.2	2.6	1.1
Catalyst (Mustang II)	3.67	8.6	1.1	----	0.16	0.3	0.4	---	0.6

[a]The methods for collecting these emissions and performing the mutagenesis bioassays have been reported elsewhere (7,8,46).

[b]The mutagenicity of the organics in the Ames bioassay is expressed in revertants/µg (rev/µg) in the mouse lymphoma bioassay as mutants/10^6 survivor/µg/ml (mut.freq./µg/ml). SCE as SCE/cell/µg/ml and skin tumor initiation as papillomas/mouse at 1 mg.

[c]The mutagenic and tumorigenic emission rates were determined from multiplying the mutagenicity of the organics times the organic emission rate to give activity emission rates per km driven.

gasoline automotive emissions to unit risk estimates for various combustion emissions or other complex mixtures through the data base established linking human, animal, and short-term genetic bioassay data. A simplified comparative approach to evaluating alternative energy sources is to employ parallel bioassay studies of the alternative (a) and conventional (c) source emissions and determine a relative risk by direct comparison as follows: increased risk (a/c) = relative bioassay potency (a/c). Because there is no *one* conventional standard petroleum-derived fuel or *one* standard combustion source, such studies need to consider the range of mutagenic and carcinogenic potency between different conventional sources and fuels. The establishment of such a range could then serve as a guide for evaluating alternative fuels or sources.

In order to evaluate a battery of short-term genetic bioassays for their utility in testing and assessment of unregulated automotive emissions, a matrix of bioassays and particle extract samples from various engines and fuels was constructed (54). Three heavy-duty vehicles, each fueled by three to five grades of diesel fuel, were operated on the '83 transient driving cycle. The light-duty automobiles were fueled by gasoline (leaded or unleaded) or the No. 2 diesel fuel. The short-term bioassays included those shown in Fig. 1 and included both mutagenesis (gene mutation, DNA damage, and chromosomal effects in procaryotes and eucaryotes), oncogenic transformation, and mouse skin tumor initiation. The mutagenesis bioassays were of three types: gene mutation in both bacterial and mammalian cells, DNA damage assays in yeast and mammalian cells, and chromosomal effects in mammalian cells. The carcinogenesis assays included oncogenic transformation in two lines of mouse embryo fibroblasts and the mouse skin tumor initiation assay in SENCAR mice. One sample from a diesel Mercedes car was tested in all 16 assays (Fig 1). The data has been compared as mutagenic emission rates.

This data base of mutagenic emission rates has been evaluated by an analysis of variance (ANOVA) method to determine if any of the emissions produced significantly different (p=0.05 level) responses (54). Because the ANOVA did not detect any significant difference with or without S9 activation, all of the results were combined for this analysis. Only the data from the following three bioassays where adequate for such ANOVA analysis: Ames *Salmonella typhimurium* assay, mouse lymphoma gene mutation assay, and sister chromatoid exchange assay in CHO cells. Among the three tests, the Ames test exhibited greatest differences between the samples as shown in Fig. 3. Samples 5 (heavy-duty Caterpillar engine) and 18 (light-duty Nissan engine), both run on minimum grade diesel fuel, had significantly higher mutagenic emission rates than the other vehicles and fuels. The two mammalian cell bioassays showed higher standard deviations, and none of the emissions were found to be significantly different, possibly due to the lack of replicate bioassays.

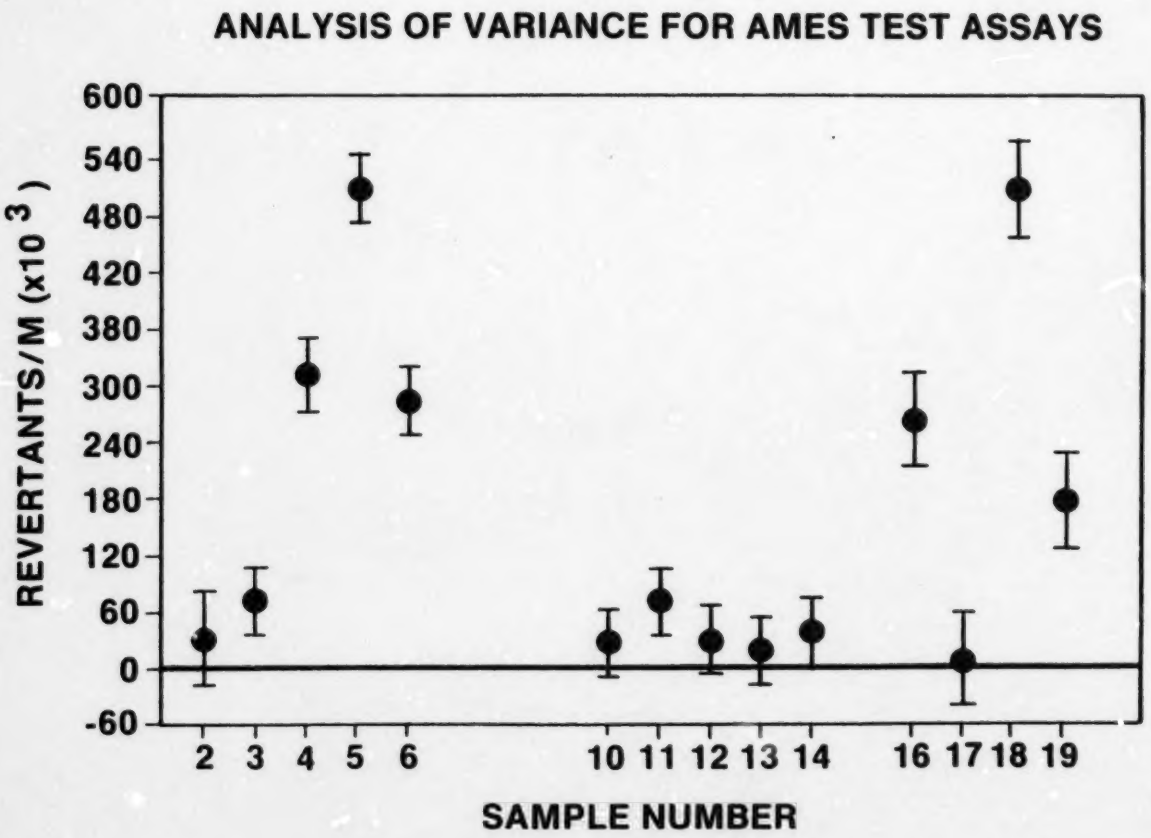

Fig. 3. Analysis of variance for Ames test assays.

The increased or decreased human cancer risk from combustion emissions may be of greater concern than the absolute risk from these emissions when we consider the effect of alternative energy technologies and fuels. The data of greatest value in determining this increased or decreased risk are comparative bioassay data on the conventional and alternative technology being evaluated. Such studies should be useful in providing direction for engineers and chemists to design alternative energy sources and fuels that result in less mutagenic and potentially less carcinogenic emissions.

The comparative potency method for cancer risk assessment described in this paper cannot be employed without uncertainty or without invoking the constant relative potency assumption inherent in the method. It is important to recognize that the assumptions and uncertainties are different from those employed in using either low-dose extrapolation techniques with human data or scaling factors with animal data. This makes it possible, therefore, to perform quantitative cancer risk assessments using more than one approach with different methodological assumptions. In the future, more information may be gained by comparing the quantitative assessments for one source using several independent cancer risk assessment methodologies.

ARE DIESEL PARTICLE EMISSIONS A SIGNIFICANT SOURCE OF HUMAN EXPOSUE TO MUTAGENS AND CARCINOGENS?

The relative contribution of different sources to ambient particle concentrations has been determined using either dispersion or receptor modeling. These studies show that combustion emissions account for most of the respirable (<2.5 um) particles in the air (55). We have recently used these methods to estimate the contribution of various combustion sources to the airborne mutagenicity due to these particles (56). Since a much larger data base is available on the Ames Salmonella assay, this data base was used for the emission factors. Table VII summarizes the findings from these studies. Using simply the information in EPA's publication of the annual U.S. fuel use by category, we estimate that 44% of the mutagenicity emitted into the air was derived from diesel vehicles, 22% from gasoline vehicles, and 32% from residential heating. Dispersion modeling was used to estimate ambient concentrations in a theoretical city and in actual cities where more detailed source inventories and meterological factors can be considered in a more complex dispersion model. In the examples shown in Table VII, 38-51% of the mutagenicity of these locations was estimated to arise from diesel vehicles. Receptor modeling uses data from ambient concentrations of tracer signature chemicals (e.g., lead for automobiles) to determine the contribution from various sources. The data shown for Denver, Colorado was derived from a study to

apportion the particles and organics using receptor modeling (57). By applying data on the mutagenicity of each of these source emissions, assuming no atmospheric transformation of the mutagenicity, only 5% of the mutagenicity was estimated to be derived from diesel trucks and 56% from gasoline vehicles. Recently we have actually used mutagenicity as a parameter for apportionment in a receptor modeling study in Albuquerque, New Mexico. Although this study was not designed to separate diesel from gasoline vehicle emissions, 50% of the mutagenicity of this air shed in wintertime was due to automotive emissions. In future studies we plan to improve our ability to directly measure the contribution of various automotive and residential heating sources to the airborne mutagenicity and tumorigenicity.

The impact of automotive emissions on the total human exposure is generally less than that estimated by considering only outdoor ambient air. Most individuals spend over 80% of their time indoors at home or work. In a recent study of ten homes, we found environmental tobacco smoke to be the principal source of indoor particle-assocoated mutagenicity (59).

TABLE VII

CONTRIBUTION OF MOBILE SOURCES AND RESIDENTIAL HEATING TO THE AIRBORNE MUTAGENICITY ASSOCIATED WITH RESPIRABLE PARTICLES

Source	Emissions from U.S. Annual Fuel Use %	Dispersion Modeling			Receptor Modeling	
		Theoretical %	Site A %	Site B %	Denver %	Albuquerque %
Mobile Sources						
Diesel	44	49	51	38	56	
Gasoline	22	22	23	17	5	
(TOTAL)	(66)	(71)	(74)	(55)	(61)	50
Residential Heating	32	24	25	43	39	50

CONCLUSIONS ON THE VALUE OF SHORT-TERM GENETIC BIOASSAYS

In retrospect, it is now safe to conclude that short-term mutagenicity assays were not only useful but instrumental in:

1) Indicating that diesel soot was potentially carcinogenic and should be evaluated on chronic animal cancer bioassays.

2) Identifying NO_2-PAHs as potential carcinogens in this very complex mixture.

3) Providing initial evidence that the mutagens were bioavailable.

4) Estimating the relative importance of various sources and fuels and other factors which can influence human exposure to carcinogens.

This is not to say that short-term bioassays used alone can accomplish all of this. However, used in combination with chemical/analytical methods and toxicological tools, short-term genetic bioassays have become a critical component of many environmental health studies.

Although substantial advances in our knowledge of the toxicology of diesel emissions have been made since 1978 when the initial observation that the organics extracted from diesel soot were mutagenic, a number of important questions remain not only for diesel emissions but for other combustion sources as well. Are the chemicals which induce positive results in the short-term bioassays the same agents which cause tumors in chronic animal bioassays? Which phase of the diesel emissions (gaseous or particulate) is carcinogenic in the animal inhalation studies?

With advances in our understanding of the molecular mechanisms involved in producing chronic effects such as cancer, it is possible that new genetic tools and short-term bioassays will continue to contribute to our ability to answer these and other questions as they arise.

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